The University of Chicago

NOTES ON THE BIOCHEMISTRY OF THE TOMATO SEEDLING

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY
JUNE, 1942

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GRANT ROGERS BARTLETT

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ACKNOWLEDGMENTS

This research was carried out under the direction of Dr. F. C. Koch and Dr. E. S. G. Barron. For their continual encouragement and advice and for the use of equipment of their laboratories the author expresses his sincerest thanks.

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NOTES ON THE BIOCHEMISTRY OF A SEED

INTRODUCTION

A seed can be considered a complete living organism. Appropriate stimulation by temperature and moisture initiates a rapid cellular multiplication giving rise to the various structures characteristic of the young plant. The seed usually contains reserve food supplies of fat, carbohydrate, and protein which serve as raw materials for the formation of various new chemicals required in the structure and function of the growing seedling and which serve also as fuel to supply the energy requirements of the different chemical conversions taking place. Usually adequate food reserves are stored within the seed to allow for a considerable growth of the plant on water alone.

This investigation was undertaken to gain some insight into the changes taking place in the growing seedling, especially into the mechanisms by which the seedling tissue utilizes its food supply. Some seeds, for example, wheat, corn and rice contain predominately starch as a food reserve. Others, such as cottonseed, peanut and sunflower contain oil as the major food reserve. Because of our meager knowledge in the field of fat metabolism an oil-bearing seed was chosen with the hope that, among other things, its activities might throw some light on the biological changes of fat. The tomato seed, an oil-bearing type, was selected for this investigation.

It has been established that associated with the growth of an oil-bearing seed there is a decrease in oil content with an increase in carbohydrate (1). It seems quite likely that the fatty acids are being converted to carbohydrate. The change in the oil content of the tomato seed during germination is shown in Table 1.

TABLE 1

CHANGE IN OIL CONTENT DURING GERMINATION

Sample	% Oil of Dry Weight Seed--Seedling
Ungerminated seed	26
2 day seedling	27
3 day seedling	29
4 day seedling	26
5 day seedling	23
9 day seedling	14
10 day seedling	8
15 day seedling	4

Oil analyses by 2 hour soxhelet extraction of dry seedling with Petroleum Ether.

The apparent slight increase in oil content during the early stages of germination is difficult to understand. This may be due to an addition of oxygen to fatty acid chains before they have broken down to a stage where they are non-extractable by fat solvents.

It was the original interest of the author to investigate the mechanism of this metabolism of oil by the seedlings. Some observations have been made along this line, but it so happens that the majority of the research has turned into apparently unrelated fields. However, it is hoped that the increase in knowledge gained in this investigation will provide a foundation for easier work on the elucidation of the oil metabolism and of any other changes occurring in the seedling.

In the remainder of this introduction some notes are given on general methods used in handling the seedlings.

The research is limited to the "seed-seedling system." By this is meant that period of growth which takes place before the complete depletion of the food reserves in the seed, and which can be attained by supplying the seed with pure water alone. The investigation was confined to a ten-day period, by which time the seedlings had grown from an original seed diameter of 3 mm to an overall length of approximately 8 cm. During this time the seedlings showed no deficiency symptoms and were apparently normal. The root first breaks through the seed coat towards the end of

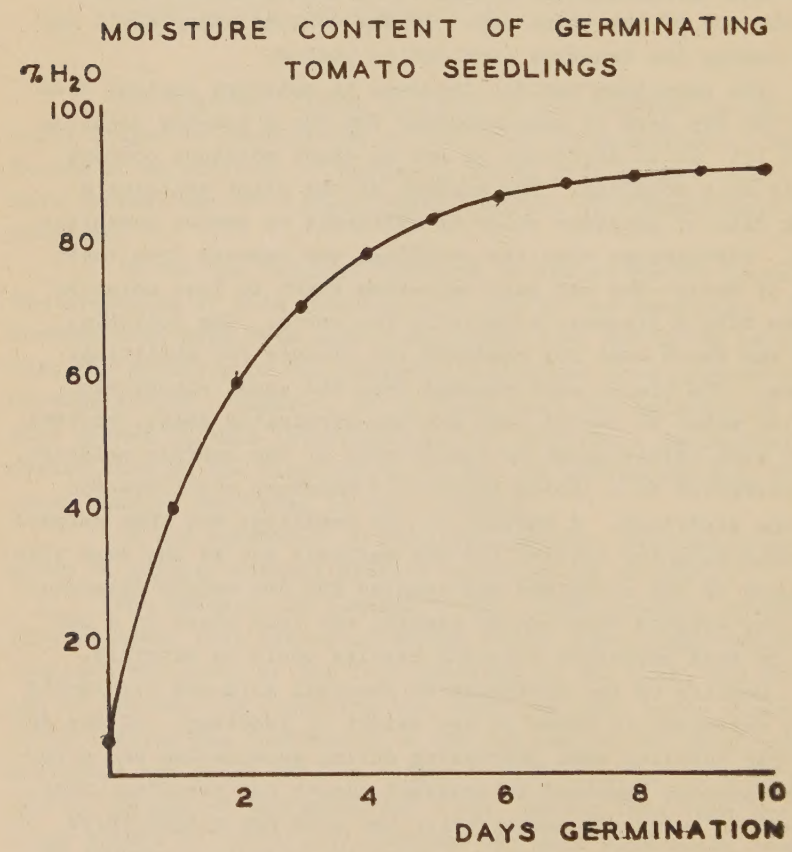
the second day of soaking in water and from that time on there is a rapid elongation of the seedling.

The seedlings were grown in glass or enamel-ware pans on washed sand, soaked with distilled water. The sand was cleaned by chromate-sulfuric acid cleaning solution, followed by thorough washing with water. The seedling pans were kept covered with glass plates. High germination tomato seeds were thoroughly washed with water and air dried before use. Varietal differences in the investigations to be reported appeared not to be significant. Seedlings were grown at 28° to 30°. Unless otherwise stated, the seedlings were grown in the dark in order to avoid possible complications due to photosynthesis. In the light, chlorophyll appeared early during the germination period--at about the end of the third day. In the dark no chlorophyll was formed during the two-week germination period.

The seedlings rapidly increase in moisture content from 5% for the dry seed to approximately 90% for a ten-day seedling (Figure 1). It is difficult to get an exact moisture content analysis on a seedling. The surface of the plant contains a varying film of moisture which is difficult to remove quantitatively. Furthermore when the seedlings are removed from their source of water--the wet sand bed--they start to lose moisture from the turgid tissues, especially the roots. The following method was found best for handling the tissues for analytical purposes. The plants were removed from the sand, rinsed with distilled water to remove sand and non-germinated seeds, blotted lightly with filter paper to remove most of the surface moisture, and transferred to a closed bottle for moisture equilibration among the seedlings. A portion of the seedlings was then weighed (in closed weighing bottle) for the analysis and at the same time an aliquot of the seedlings was removed for dry weight determination. Dry weights were run by heating for four hours in a 100° oven. By this procedure constant results could be obtained.

Results on the synthesis of ascorbic acid and carotenoid will be expressed in terms of dry weight of seedling. If the dry weight per seedling were decreasing during germination and a particular compound remained in constant amount per seedling, then, expressed in terms of concentration per gram dry weight there would be an apparent synthesis. There is actually a gradual decrease in dry weight during the germination period from 2.7 mg

FIGURE 1


MOISTURE CONTENT OF GERMINATING TOMATO SEEDLINGS
% H_2O
100
80
60
40
20
2
4
6
8
10
DAYS GERMINATION

for an ungerminated seed to 2.3 mg for a ten-day seedling. This correction factor must be kept in mind in interpreting changes of concentration of any substance during the germination.

Much use of the Potter-Elvejhem type of glass test tube homogenizer (2) was made for the various extractions (thiamin, carotenoid, ascorbic acid) and for the preparation of seedling suspensions for respiration studies. The latter were always made by homogenizing in an ice bath. Easier disintegration of the tough seed coat was made possible by placing short jagged glass protrusions on the bottom of the homogenizing cylinder.

This investigation has taken the course of following the presence and changes of certain known biocatalysts which might conceivably play an important role in the physiological changes occurring in the seed-seedling system. These include studies on carotenoid, ascorbic acid, sterol, tocopherol and thiamin. In addition notes are given on the respiration of the seedlings.

NOTES ON THE BIOCHEMISTRY OF A SEED

RESPIRATION

A few preliminary observations have been made on the respiration of the tomato seedlings. Manometric experiments were performed in the usual Warburg Equipment.

Oxygen Uptake and R.Q. of Seedling Suspensions

In the experimental set-ups each vessel contained 2.0 cc of seedling suspension (approximately 50 mg dry weight of tissue) in 0.05 M phosphate buffer, pH 6.4. Vessels for oxygen uptake contained 0.15 cc 15% KOH in center cup. Vessels for initial and final CO_2 dissolved in the medium contained 0.3 cc 2N HCl in side arms. Temperature of the analysis 28°. R.Q. calculations by the method of Warburg and Yabusoe (3).

Finely homogenized suspensions of seedlings exhibit a fairly rapid uptake of oxygen. The QO_2 for a large number of determinations averaged around 2. (QO_2 = cmm O_2 per mg tissue [dry] per hour.) This represents the whole seedling tissue. Cell suspensions have been prepared with the inert seed coats and cellulose material removed which show a much higher respiratory activity. Variations in QO_2 values depended chiefly on the time elapsed between crushing of the tissues and the taking of readings, for the oxygen uptake of the seedling suspension shows a gradual falling off with time. See Table 3.

Associated with the oxygen consumption of seedling tissue is a relatively small CO_2 evolution, giving R.Q. values of approximately 0.3. The low R.Q. could be explained if a large percentage of the metabolism of the seedling involved the conversion of fat to carbohydrate.

Oxygen Uptake and R.Q. of Intact Seedlings

It seemed advisable to check whether intact growing seedlings would give the same type of respiratory exchange as that

found with homogenized seedling suspensions. Tomato seeds were grown on cotton in Warburg vessels: 8 seeds per vessel on 150 mg of cotton soaked with 3.0 cc water, 0.15 cc 15% KOH in center cups for oxygen uptakes, incubated in constant temperature bath at 30°. Seedlings grew apparently normally in this set-up and there was no sign of bacterial or mold growth at the end of the experiment. Readings were taken at various intervals for five days.

For a day and a half after the start of soaking of the seed there is a steady slow oxygen uptake with an R.Q. of approximately 1. Towards the end of the second day the oxygen uptake increases reaching a value of $QO_2 = 1$. Meanwhile the R.Q. starts falling. (It is at the end of the second day that the first visible signs of growth appear with the breaking of the root tip through the seed coat.) During the first part of the third day the QO_2 has risen to 2 and by the end of the third day the QO_2 is approximately 3 and the R.Q. has fallen to a value of approximately 0.3. These values are maintained throughout the remainder of the experimental period.

Therefore, intact seedlings maintain an oxygen consumption at a somewhat higher rate than that observed with the seedling suspensions. The low R.Q. found for the seedling suspension is confirmed by the studies on intact seedlings.

Hydrogen Ion and Seedling Respiration

pH effects on the oxygen uptake of seedling suspensions were run in phosphate buffers (0.05M) between pH 5.43 and 6.73. A gradual increase in oxygen uptake is observed with increasing pH between these limits. It is noted that the seedling extracts themselves are highly buffered around pH 5.9. Table 2.

TABLE 2

RELATION OF pH TO OXYGEN UPTAKE IN SEEDLING SUSPENSIONS

pH Pure Buffer	pH Buffered Seedling Suspension	2.0 cc Seedling Suspension in 0.05 M Phosphate Buffers, 0.15 cc 15% KOH in Center Cup. cmm O_2/30 Minutes
4.50	5.43	170
6.46	6.00	202
6.95	6.23	267
8.51	6.73	274

Temperature and Seedling Respiration

Temperature effects on the oxygen uptake of seedling suspensions were determined between 4° and 48°. The oxygen uptake increased to 36° followed by a rather sharp inactivation at increasing temperatures. Figure 2. The temperature coefficient Q_{10} from 4° to 28° was 1.85. This is of the same order of magnitude as that usually found for the temperature coefficient of cellular respiration. The sharp decline observed above 38° was undoubtedly due to protein denaturation.

Cytochromes and Seedling Respiration

Most of the oxygen consumption of animal tissues appears to pass through the cytochrome system, respiration being almost completely inhibited by small concentrations of cyanide which block out the cytochrome oxidase. Cyanide inhibition experiments were performed on the tomato seedling suspensions, care being taken to avoid distillation of HCN to the KOH containing cup. Table 3. During the first half hour of the experiment inhibition of oxygen uptake was only 31%, with as much as 0.01M HCN. The extent of the inhibition increases with time. It appears from these results that at least a good percentage of the oxygen consumption by the seedlings is not catalyzed by the cytochrome system.

TABLE 3

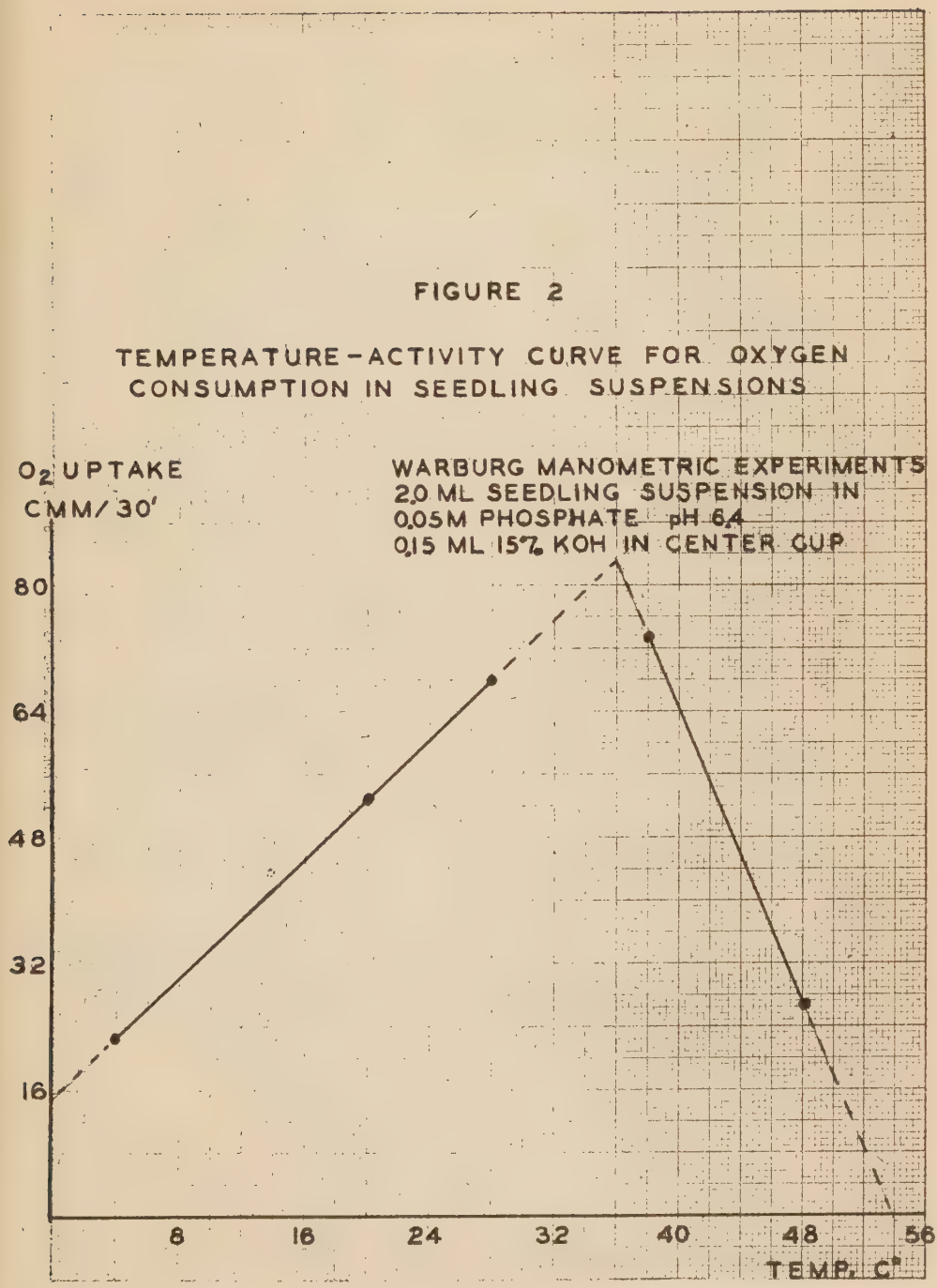
CYANIDE INHIBITION OF SEEDLING RESPIRATION

2.0 cc Seedling Suspensions in 0.05 M Phosphates pH 6.4
0.15 cc KOH--Center Cups (for cyanides, adjusted with KCN to prevent absorption of HCN)

Period of Experiment	Control cmm O_2	0.01 MCN--Final Conc.		0.001 MCN--Final Conc.	
		cmm O_2	% Inhib.	cmm O_2	% Inhib.
1st ½ hr.	89.4	61.3	31.4	67.1	25.0
2nd ½ hr.	39.6	21.4	46.0	25.2	36.4
Next 1 hr.	55.6	19.9	64.2	27.3	50.9
Next ½ hr.	23.6	5.5	76.7	8.0	66.1
Total 2½ hr.	208	108	48.0	128	38.5

FIGURE 2

TEMPERATURE-ACTIVITY CURVE FOR OXYGEN CONSUMPTION IN SEEDLING SUSPENSIONS



Press juice emulsion of the tomato seedlings when made 0.01 M with cyanide or treated with bisulfite and examined spectroscopically failed to show evidence of the 550 mu band of reduced cytochrome c, nor was added reduced cytochrome c oxidized by the suspension, which would mean that there is no cytochrome oxidase. Therefore, the cytochrome system, if present at all, must exist in the tomato seedling in very small concentration. On the other hand, extracts of wheat germ showed a distinct band for reduced cytochrome c after addition of a small amount of bisulfite.

Succinic-dehydrogenase and Seedling Respiration

In animal tissues, a large portion of the cell respiration (that concerned with carbohydrate oxidation) seems to be catalyzed by the oxidation-reduction system succinate-fumarate. Experiments were carried out to determine the presence of this catalysis in the tomato seedling. Evacuated seedling suspensions in Thunberg Tubes did not decolorize methylene blue in the presence of added succinate after incubation for four hours at room temperature. This apparently rules out succino-dehydrogenase as an accessory catalyst of oxidation in these fatty tomato seedlings. However suspensions of wheat and corn seedlings fairly rapidly decolorized methylene blue in vacuo in the presence of added succinate.

Comparison: Wheat and Tomato Seed

It can be pointed out here that three major differences have been found between the fat-type, tomato seed and the starch-type, wheat; in cytochrome and succinic dehydrogenase contents (above), and in degree of pyruvate utilization. (See page 16.)

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THIAMIN

Through its vitamin action thiamin has been shown to be essential for the normal functioning of the animal organism. Demonstrated methods of action of this compound involve the catalysis of various reactions of pyruvic acid (4). Only the diphosphorylated form has been shown to be active. This activity assumes great importance because of the role of pyruvic acid as an intermediate in the transformations of foodstuffs within the cell. Thiamin is not exclusively of importance to the animal cell. Different microorganisms require diphosphothiamin (DPT) for reactions involving pyruvic acid. An essential step in the alcoholic fermentation by yeast is the decarboxylation of pyruvic acid, catalyzed by DPT. Thiamin has been shown to be necessary for the growth of isolated plant roots (5).

Thiamin is widely distributed throughout the plant kingdom (6). It seems worthwhile to consider whether it plays a role in the metabolism of plant cells similar to any of those which have been demonstrated for animals and microorganisms.

In the following section the presence of thiamin and its phosporylated form are investigated quantitatively in the tomato seed-seedling system and experiments are performed to throw some light on its physiological function in the seedling.

THIAMIN -- EXPERIMENTAL

Total Thiamin Concentration in Seeds and Seedlings

Methods

Analyses were carried out by the Hennessy and Cerecedo thiochrome procedure (7) as modified by the Committee on Vitamin Analyses, American Chemical Society, 1941 (8). The method as used on the seedlings is as follows. Approximately one gram (as dry weight) of fresh seedlings is thoroughly disintegrated in a large (100 cc) test tube homogenizer with 40 cc 0.1 N H_2SO_4. A reflux condenser is attached to the homogenizer tube and the extraction carried out in a boiling water bath for 1/2 hour with frequent shaking. After cooling 150 mg of takadiastase (Parke Davis) in 2.5 cc 2N sodium acetate is added and the mixture transferred to a 50 cc volumetric flask and made to volume. The volumetric flask is incubated at 45° for two hours for conversion of the diphosphothiamin to free thiamin. The extract is then filtered and 15 cc aliquots passed through a 0.7 x 10 cm column of 60-80 mesh decalso (Permutit Co.). After washing with water the adsorbed thiamin is eluted with 25% KCl, the eluate being brought to 25 cc. 5 cc aliquots of the KCl eluate are oxidized with 3 cc 15% NaOH containing 0.03% potassium ferricyanide and the resulting thiochrome extracted with 15 cc isobutanol. A blank is run on the KCl eluate without ferricyanide. Checks on the efficiency of the oxidation and extraction and also for standardization of the fluorometer are run by addition of known amounts of pure thiamin to an aliquot of the KCl eluate. Recoveries of pure thiamin by the whole procedure amount to 94 to 100%.

Readings of thiochrome concentrations were made with a photoelectric fluorometer devised in this laboratory with the cooperation of Dr. R. Abrahms of the Chemistry Department. Characteristics of the instrument are: constant output Hanovia G.E. Lamp for ultraviolet source, corex filter between U.V. and sample for removal of visible light, yellow filter between sample and photocell for removal of possible non-blue extraneous fluorescence, amplified photocell current balanced on Type K Leeds and

Northrup Potentiometer, sample cells constructed to contain from 5 to 10 cc total volumes. Straight line variations of voltage change were obtained with 0.03 to 3.0 gamma per cc concentrations of thiochrome. The instrument gives an increase of 192 millivolts for an increase in concentration of thiochrome of 0.1 gamma per cc in isobutanol.

Results

The thiamin concentration in the tomato seed was found to be unusually high--17 gamma per gram. For comparison, the wheat grain, considered a rich source of thiamin in the plant kingdom, contains from 3 to 5 gamma per gram. The concentration of total thiamin in the tomato plant gradually decreases during the germination, reaching a value of 12 gamma per gram after 10 days of germination. Table 4.

TABLE 4

THIAMIN CONTENT OF GERMINATING TOMATO SEEDLINGS

Sample	Total Thiamin gamma/gram dry weight
Ungerminated seed	17
2 day seedling	16
5 day seedling	15
7 day seedling	13
10 day seedling	12

The high concentration of thiamin in the tomato seed was confirmed by bioassay. Analyses were run by the rat curative technique as given by the U.S.P., 2d Supplement 1940. Averages of ten analyses gave a thiamin value of 16 gamma per gram.

Diphosphothiamin

Since only the diphosphorylated form of thiamin appears to be functionally active in tissue metabolism, it is of interest to follow the presence of this compound in the seedlings.

Methods

DPT was analyzed manometrically by its cocarboxylase

action in the decarboxylation of pyruvic acid, using alkaline washed yeast as the enzyme source. Details were according to the procedure given by Ochoa and Peters (9). In the preparation of the sample a portion of fresh seedlings was homogenized in water, heated for 3 minutes at 100°, and an aliquot taken for dry weight determination. The mixture was then centrifuged and 0.5 cc portions of the supernatant used in the manometric tests. In each analysis comparisons were made with three concentrations of pure DPT and the concentration of the unknown interpolated from the standard curve obtained. Fairly straight line variations of CO_2 output and DPT concentration were obtained between 0.04 and 0.2 gamma DPT per vessel. In setting up the experiment each vessel contained 1.0 cc alkaline washed yeast in 0.1 M phosphate pH 6.2, 0.5 cc of sample or standard DPT in water, 0.1 cc = 10 ɣ Thiamin, 0.1 cc = 0.1 mg Mg and Mn, 0.2 cc = 2 mg Na pyruvate (in side arm).

Results

Only fair checks were obtained with successive determinations of DPT. However, extracts of the ungerminated seeds gave no decarboxylation while three-day and five-day seedlings showed definite cocarboxylase activities giving values ranging between 1 and 1.5 gamma DPT per gram dry weight of seedling. Thus it can be concluded that at the start of growth thiamin is phosphorylated by the seedling although it appears that in the growing plant the diphosphothiamin remains only a small proportion of the total thiamin content.

Pyruvic Acid Metabolism

Methods

Samples: homogenized seed or seedling suspensions in 0.05 M phosphate, pH 6.4.

Manometric Experiments: Warburg microrespirometers, analyses at 28°.

Pyruvate Analyses: CO_2 production in presence of washed yeast, acetate-phosphate buffer pH 4.73, 25°.

Acetaldehyde Analyses: Low temperature (60°) steam distillation of acetaldehyde from sample into excess bisulfite, using modified Parnas apparatus. Bound bisulfite according to iodine titration procedure of Friedemann (10).

Results

Analyses for pyruvate after incubation of known amounts of Na pyruvate with seedling suspensions show that a small amount of this compound is used up by the tissue. Manometric experiments demonstrate that the pyruvate utilized can be accounted for by the increased CO_2 production in the presence of pyruvate, that is, a straight decarboxylation. Analyses for acetaldehyde and pyruvate after incubation of seedling suspensions with pyruvate give values corresponding to a 46% conversion of pyruvate to acetaldehyde. From the results of the manometric experiments mentioned previously it seems likely that at least the major portion of the pyruvate is being further metabolized. If this is so, it seems that the metabolism of the acetaldehyde is non-oxidative in character because of the lack of increased oxygen uptake in seedlings with added pyruvate. Of course there is the possibility that part of the residual seedling respiration is depressed in the presence of added pyruvate. See Table 5.

Synthesis of DPT and Carboxylase by Seedlings

Suspensions of ungerminated seeds do not decarboxylate pyruvic acid. This would be expected from the fact that no DPT was found in the ungerminated seeds. A further question of interest is whether the carboxylase enzyme is synthesized at the start of growth. After additions of DPT to suspensions of dry seeds the resulting mixture will decarboxylate pyruvic acid. Three-day seedling suspensions which decarboxylate pyruvic acid and contain approximately one gamma per gram dry weight of DPT do not give additional decarboxylation of pyruvate with added DPT. Table 6. Therefore, one can conclude: first, that carboxylase is present in the ungerminated seed and, second, that the carboxylase in the seedling is saturated in respect to DPT.

Comparison with Wheat and Corn

A large part of the metabolism of the tomato seedlings must involve transformations of fats. It seemed worth while to see whether a seedling whose matabolism is chiefly concerned with carbohydrates would differ from the tomato seed in its utilization of pyruvic acid. It was found that wheat and corn contain

very active pyruvate decarboxylating systems. This activity is concentrated in the germs of the grains and is of a similar order of magnitude to that of an active yeast. Because of the comparatively small amount of pyruvate utilization by the fatty tomato seedling it is probable that the metabolism of the fat in this case does not pass through pyruvic acid.

TABLE 5

PYRUVATE UTILIZATION AND ACETALDEHYDE PRODUCTION
BY TOMATO, WHEAT, AND CORN

Sample	Millimoles Pyruvate Utilized per gram Tissue (dry wt.) per Hour	% Pyruvate Utilized Recovered as Acetaldehyde
Ungerminated tomato seed	0	0
Tomato seedling a) Extra CO_2 (manometrically as pyruvate	0.029	
b) Yeast pyruvate analysis	0.030	46
Wheat germ	0.20	--
Corn germ	0.22	105

TABLE 6

SEED TO SEEDLING
CARBOXYLASE AND DPT CONVERSIONS

Sample	Extra CO_2 from added Pyruvate - cmm/2 Hours	
	Control	50 gamma DPT
Ungerminated seed (100 mg)	0	61
3 day seedling (85 mg)	58	53

Samples in 2.0 cc 0.05 M Phosphate, pH 6.4
0.02 millimoles pyruvate added.

* * * * *

Since this work was initiated, papers have appeared demonstrating the presence of carboxylase systems in the pea (11) and barley (12).

NOTES ON THE BIOCHEMISTRY OF A SEED

ASCORBIC ACID

As a basis for understanding the changes taking place in this seed-seedling system, in particular the metabolism of the stored fat, it does not seem amiss to focus attention on ascorbic acid. This compound has been adequately proved a vital necessity for the functioning of the animal organism. Its very widespread occurrence in active plant cells suggests that it plays a similar important function in plant tissues. One is struck by the fact that, although the dormant seed contains no trace of ascorbic acid, immediately with the start of cellular activity, a considerable synthesis of the compound occurs. It seems unlikely that the ascorbic acid is merely an intermediate in the breaking down or synthesis of some other compound.

Possible Functions of Ascorbic Acid

Notwithstanding a large number of papers on the subject containing innumerable speculations, Stiebling could correctly state in a recent review (13) "in spite of voluminous research little light has been thrown on the physiological role of ascorbic acid."

Ascorbic acid-dehydroascorbic acid is a sluggish oxidation-reduction system with an E_h of .100 pH 6 (14). The ascorbic acid has been assumed to play a role as a catalyst in biological oxidations and reductions, by a number of writers. However, all work to prove this point has been negative. Barron in 1938 (15) summarized the situation to that date by saying: "There is no evidence that ascorbic acid takes part in biological systems as an oxidation-reduction catalyst." Since that time there has been no change in the situation. There have been numerous negative attempts to show that ascorbic acid affects the respiration of deficient guinea pig tissues (16). Positive reports on tissue

respiration effects (17), for example, Quastel (18) and Zilva (19) have been so inconsistent and of such a small order of magnitude that for the present one must assume that there are no major effects. Furthermore present evidence favors the view that ascorbic acid exists normally both in plant and animal tissues, not in equilibrium with the dehydro form, but exclusively in the reduced state.

Ascorbic Acid and Hydrolytic Enzymes

The evidence on this subject is difficult to interpret. It has been maintained by one group of workers that ascorbic acid exerts a marked activating effect on lipases, invertase and other hydrolases, and some would even have this activating effect as the chief physiological function of the compound. However, another group of workers have claimed that ascorbic acid not only had no stimulating effect on these hydrolytic enzymes, but actually inhibited their activity.

Tauber reviewed this subject in 1938 (20). Since that date there has appeared considerably more conflicting evidence. It seems that under certain conditions ascorbic acid may accelerate an enzymic reaction. However, in no instance has it been shown that the compound is an essential coenzyme for any particular enzyme system. It has been postulated that its stimulating effect may be due to the maintenance of a more favorable potential for the reaction in question.

Ascorbic Acid, Carotenoids and Chlorophyll

It has been often proposed by plant biochemists that the functions of ascorbic acid, carotenoids, and chlorophyll, and therefore of the photosynthetic process, are closely linked (21). The evidence is based solely on the analytical distribution of these compounds in plant tissues. A number of papers have pointed out that with certain exceptions (especially fruits) chlorophyll containing tissues are richer in ascorbic acid. However, the differences generally speaking are small and at this stage of our knowledge on the subject could just as well be explained as being due to a higher metabolic level of the green tissue rather than indicating a specific chlorophyll relationship. Giroud, an ex-

ponent of an ascorbic acid-photosynthesis relationship believes that the silver nitrate reducing reaction of the chloroplasts is due to a high concentration of ascorbic acid in this part and further demonstrates a relationship between ascorbic acid and the photosynthetic mechanism (22). Similarly there have been papers emphasizing that tissues high in carotenoids are high in ascorbic acid (23). However, this correlation is by no means general (24). Von Euler's suggestion (25), often repeated by others, that ascorbic acid in tissue is protected by the antioxidant activity of the carotenoids, is without experimental foundation. There is no evidence that these pigments can act catalytically as antioxidants. If chlorophyll, carotenoid and ascorbic acid concentrations happen to be high in a tissue of high active metabolism, this does not necessarily mean that their functions are closely related or linked.

Biological Synthesis of Ascorbic Acid

Ascorbic acid is synthesized in both plant and animal tissues. There have been numerous papers with much speculation on the mechanism of the synthesis. In plant tissues, it has been claimed and denied that light is an important factor in synthesis of ascorbic acid (26). On substrate deficient tissues ascorbic acid formation has been demonstrated from added sugars, especially mannose (27). Japanese workers have claimed that mannose is the precursor of ascorbic acid in both plant and animal tissues (28). However, many other workers have been unable to find increased ascorbic acid synthesis from various sugars including mannose (29). If we assume that sugars can give rise to ascorbic acid, we still are entirely in the dark as to the intermediate steps involved. Furthermore, it is possible that the carbon chain of the hexose does not pass intact to the ascorbic acid but that the latter is built up from carbohydrate degradation products. Rudra (30) claims that manganese is necessary for ascorbic acid synthesis. His experiments show a slightly increased synthesis in seedlings on addition of manganese. He gives inadequate proof that this is not an unspecific salt effect. Moreover, his results could be explained by a general stimulation of tissue activity by this essential element in partly manganese deficient seeds. This matter, as do other problems in the field, awaits

the separation of isolated ascorbic acid synthesizing enzyme systems. Longenecker et al (31) have carried out some interesting experiments on ascorbic acid excretion in nutritionally deficient rats. The ascorbic acid excretion (a measure of the synthesis) was markedly increased by a non-glyceride lipoid extract of oats. Further experiments showed that a large variety of more or less unrelated compounds gave similar results. Interpretation of these facts at the present time is difficult. As the authors suggest, the compounds probably do not act as precursors of ascorbic acid but in some way may stimulate the ascorbic acid synthetic mechanism.

State of Ascorbic Acid in Tissues

Some workers in the field have felt that ascorbic acid and the oxidized dehydro form exist in tissues in a state of equilibrium and that normally considerable quantities of the latter are present in some cases. At the present, it seems most likely that ascorbic acid exists naturally in non-senescent tissues only in the reduced state. Analyses to the contrary are probably due to not taking adequate precautions to prevent oxidation, enzymatically or otherwise, during the enalysis. A less settled argument is that concerning the existence of a protein bound ascorbic acid. Reedman and McHenry (32) and Holtz and Walter (33) have studied this in detail and conclude that there exists a combined form which is not analyzed by the standard acid extraction and dye titration method, but which can be released for titration by acid or proteolytic hydrolysis. Rujita and Ebihara's recent investigations (34) do not support this view. Many others have been unable to verify the existence of a protein combined ascorbic acid. The latter workers claim that if ascorbic acid exists in combination in living tissues, it must be a labile union which is broken by the methods of acid extraction used in the standard analytical techniques.

When ascorbic acid appears in the newly germinating seedling, it has been assumed that it has been synthesized rather than released from some combination existing in the dormant seed. This assumption is reasonable from the standpoint that the dry seed is not biologically active as an antiscorbutic food.

Ascorbic Acid Oxidase

Szent-Gyorgyi first pointed out that his hexuronic acid was catalytically oxidized by plant tissue extracts (35). After much research, it became evident that in addition to unspecific catalyzed oxidations of ascorbic acid by plant tissue extracts--copper and indirectly by phenolases and peroxidases--that there exists widely distributed throughout the plant kingdom an enzyme which specifically catalyzes the dehydrogenation of ascorbic acid (36). It does not seem to be present in animal tissues. The co-existence of appreciable amounts of this enzyme and large amounts of reduced ascorbic acid in living tissues has not been explained. It has been claimed that glutathione reduces ascorbic acid in the presence of a specific anzyme (37). The physiological role of these enzymes is difficult to interpret at the present time.

* * * * *

The following experimental part of this investigation has some bearing on most of the points discussed above.

ASCORBIC ACID -- EXPERIMENTAL

Ascorbic Acid Estimation

Analyses were made by reduction of the dye 2, 6 dichlorphenolindophenol. Photometric determinations were carried out in the Evelyn Colorimeter using the refinements proposed by Bessey (38). The technique made possible differentiation between ascorbic acid and slower reducing substances and also allowed for analyses on slightly cloudy extracts.

Analyses for dehydroascorbic acid were made by H_2S reduction according to details given by Bessey (38), with thorough removal of H_2S with wet nitrogen.

Method of Extraction

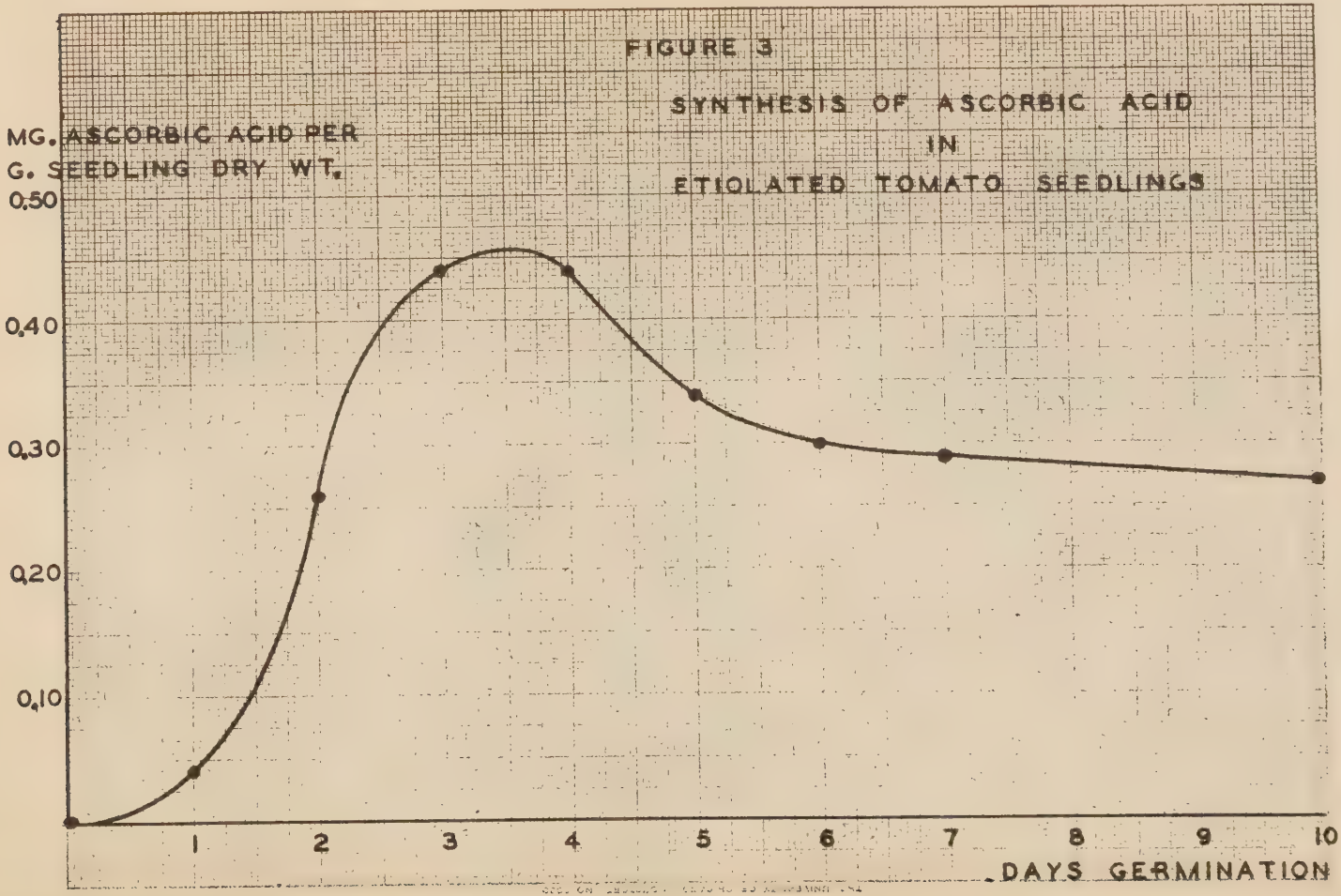
A procedure was developed which prevented all oxidation of ascorbic acid. Fresh seedlings were immediately covered by fresh 3% metaphosphoric acid in a 40 cc total volume test tube homogenizer. The seedlings were then thoroughly disintegrated using a high speed motor on the cylinder of the homogenizer. The acid extracted and stabilized the ascorbic acid as fast as the tissue was broken. The extract was then filtered and finally centrifuged to remove traces of suspended solids. An aliquot was buffered to pH 3.5 and used for the colorimetric estimation with the dye. Concentrations of pure ascorbic acid from 3 to 30 gamma per 8 cc in metaphosphporic acid-citrate gave straight line variation in the photometer with a K value of 0.088. Analyses reported are those obtained from colorimetric readings 15 seconds after mixing of the reagents.

Ascorbic Acid Synthesis in Tomato Seedlings

Etiolated seedlings were grown and prepared as previously described (Introduction). Analyses for ascorbic acid were made at various times during the course of germination. The data obtained are given in Table 7 and Figure 3. In consideration of these results a number of comments can be made:

FIGURE 3

SYNTHESIS OF ASCORBIC ACID IN ETIOLATED TOMATO SEEDLINGS



1. There is no ascorbic acid present in the ungerminated seed. Attempts with various hydrolytic procedures have failed to reveal the presence of any combined ascorbic acid at this stage.
2. After 24 hours soaking in water, until the end of the third day, there is a very rapid synthesis of ascorbic acid. The values then level off and gradually decline.
3. The loss of ascorbic acid following the fourth day does not involve replacement by the oxidized dehydro form as will be shown below.
4. The level of ascorbic acid attained in the seedling is comparable with the highest concentrations of this compound found in various plant and animal tissues.

Light and Ascorbic Acid Synthesis

The presence of chlorophyll, with the metabolism associated with photosynthesis, does not effect the occurrence of ascorbic acid in the seedlings. The green seedlings grown in the light contained approximately the same concentration of this compound as those grown in the dark. Table 8.

TABLE 8

SYNTHESIS OF ASCORBIC ACID BY TOMATO SEEDLINGS

Days Germination	mg Ascorbic Acid/gram Dry Seedling	
	Light	Dark
0	0	0
4	0.46	0.44
7	0.28	0.29
10	0.26	0.28

Slow Reduction of Dye in Ascorbic Acid Analysis

One of the difficulties in the ascorbic acid analysis by the indophenol method on animal fluids is the presence of non-ascorbic acid dye reducing substances--chiefly sulfhydryl groups.

Ascorbic acid is differentiated from these substances by means of its much more rapid reduction of the dye. It has been observed that most plant extracts do not contain appreciable amounts of such materials. These slow reducing substances are formed during the germination period, for the ungerminated seed shows no reduction of the dye. Typical time curves of the reduction are given in Figure 4. It is probable that this phenomenon is chiefly due to the presence of glutathione which is known to be present in significant quantities in seedlings and which gives a similar rate of reduction of the indophenol. The rate of the slow reduction increases slightly from the first till the seventh days of germination.

Bound Forms of Ascorbic Acid

The seedling-3% metaphosphoric acid homogenized suspension was hydrolyzed with 1% HCl according to the procedure used by McHenry (34) to demonstrate combined ascorbic acid. A 3% metaphosphoric homogenizate of seedlings was made 1% with HCl. An aliquot was taken for ascorbic acid analysis. Another portion was heated on a boiling water bath for one hour under nitrogen and then analyzed for ascorbic acid. Both samples were then treated with hydrogen sulfide for dehydroascorbic acid analysis. A similar experiment was carried out on ungerminated seeds. There was no evidence of combined acid of the type postulated by McHenry and others either in the germinating seedlings or the ungerminated seeds. Table 9.

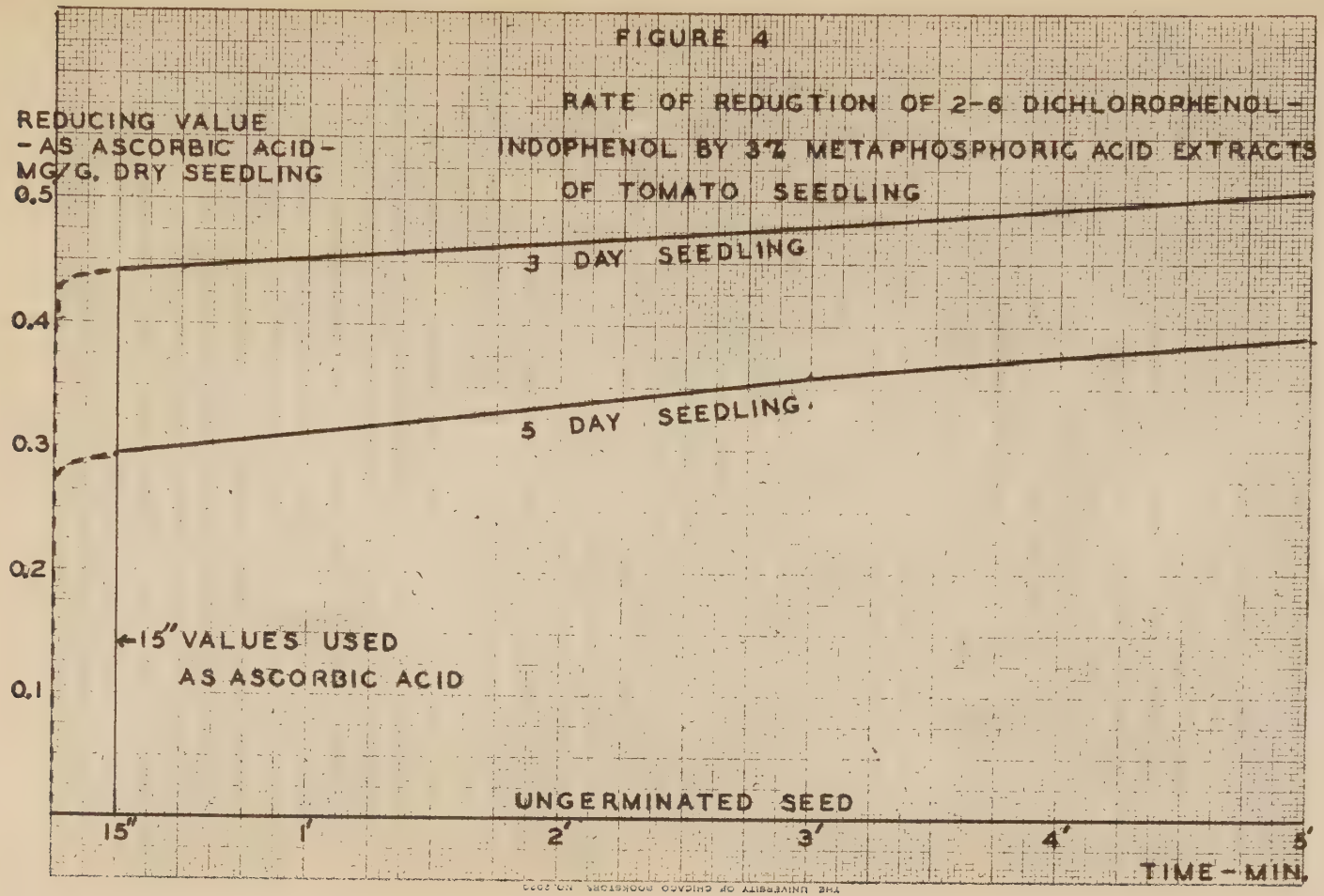
TABLE 9

COMBINED ASCORBIC ACID

Sample	Mg Ascorbic Acid/Gram Dry Seedling	
	Regular	After H_2S Reduction
Metaphos. Acid Ext. 5 day etiol. seedling in 1% HCl	0.41	0.41
Same heated 1 hour at 100°	0.40	0.38

FIGURE 4

RATE OF REDUCTION OF 2-6 DICHLOROPHENOL-INDOPHENOL BY 3% METAPHOSPHORIC ACID EXTRACTS OF TOMATO SEEDLING



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Oxidation of Ascorbic Acid by Seedling Tissue

As pointed out previously, fresh seedlings must be disintegrated in the presence of metaphosphoric acid for true values of reduced ascorbic acid to be attained. For example, a portion of seedlings was thoroughly crushed in the bottom of the test tube homogenizer and allowed to stand for 15 minutes. The crushed tissue was then covered with 3% metaphosphoric acid and extracted as usual. All the reduced ascorbic acid had disappeared.

Experiments were carried out to further investigate this change of ascorbic acid in crushed tissue--to determine whether it involved an oxidation and if so whether or not this process was enzymatically catalyzed.

Oxidation of ascorbic acid by seedling tissue was tested manometrically in the Warburg Apparatus. Preliminary experiments

TABLE 10

OXIDATION OF ASCORBIC ACID BY TOMATO SEEDLING TISSUE

Time (Minutes)	cmm O_2 Consumed		
	a	b	c
0	0	0	0
3	0	27.6	0
6	0	96.0	1.6
10	0	138	9.4
18	0	228	21.9
25	0	258	31.3
30	0	268	39.1
38	0	268	50.0
48	0	268	62.7
60	0	268	80.0

See Figure 5 for graphic analysis of oxygen consumption.

Experimental Conditions

Temperature 28°.
Sample, 5 day acetone defatted etiolated seedling powder.
Center cup all vessels, 0.15 cc 15% KOH.

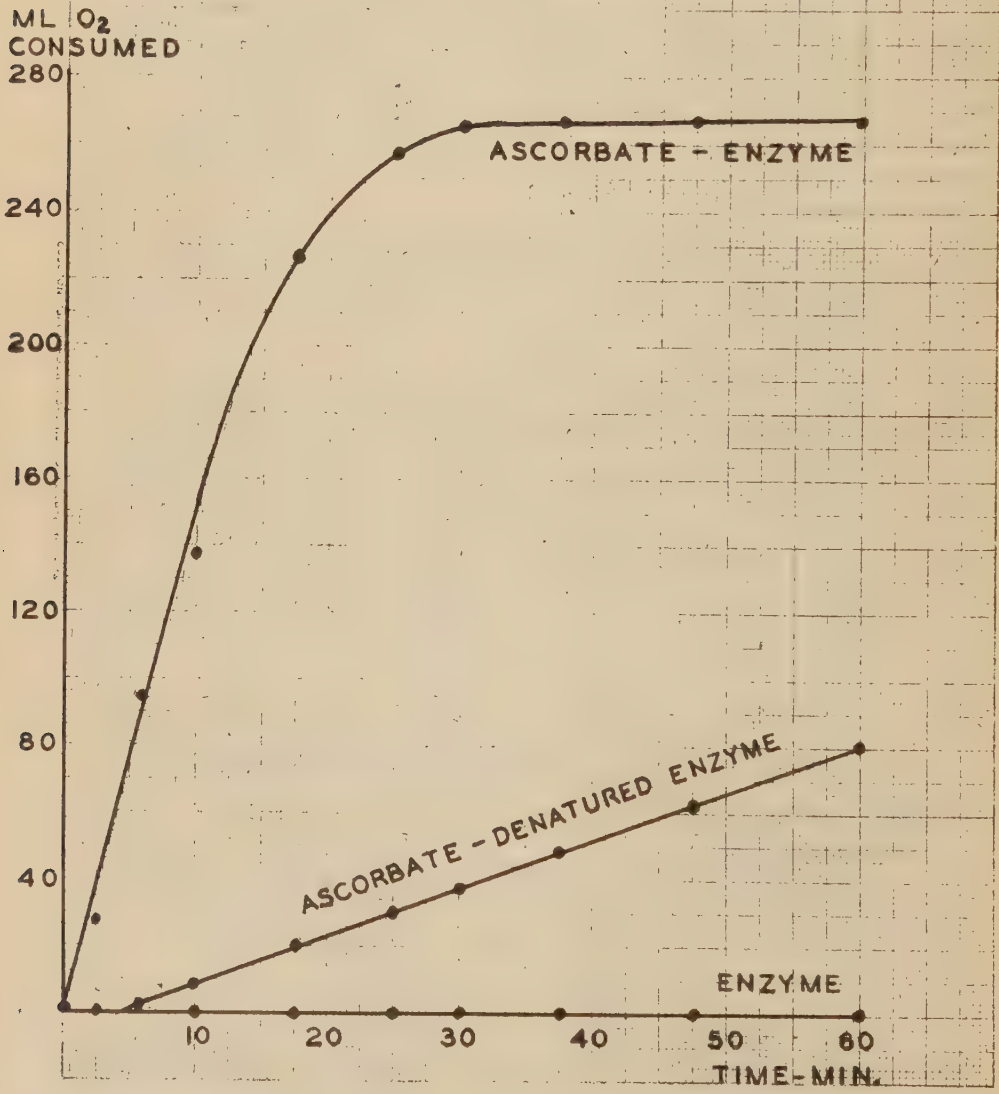
a) Blank. 25 mg seedling powder in 2.75 cc H_2O.

b) 25 mg seedling powder in 2.5 cc H_2O.
Side arm: 0.02 mM Ascorbic Acid in 0.25 cc H_2O.

c) 25 mg seedling powder in 2.5 cc H_2O.
Heated at boiling water for three minutes.
Side arm: 0.02 mM Ascorbic Acid in 0.25 cc H_2O.

FIGURE 5

OXIDATION OF ASCORBIC ACID
BY TOMATO SEEDLING ACETONE POWDER



showed that dry acetone defatted seedling powders, from which the seed hulls had been removed, rapidly oxidized added ascorbic acid. This seedling powder preparation was used because of its stability, ease of handling and because it gave zero blank ozygen consumptions.

An exactly similar experiment using acetone defatted powder from ungerminated seeds gave no oxygen consumption with ascorbic acid.

Interpretation

Barron (39) has shown that traces of copper rapidly catalyze the oxidation of ascorbic acid. With work on ascorbic acid oxidase, it has been difficult to differentiate adequately between unspecific copper ion catalyzed oxidation and enzymatic oxidation. In the above simple experiment it appears reasonably certain that the difference between the oxygen consumption of the heated and non-heated preparations represents protein catalyzed oxidation. The oxygen consumption of the heated preparation is probably due to traces of copper ion. In distilled water the addition of ascorbic acid to the seedling suspension brought the pH to 4.2. At this low pH non-specific cupric ion oxidation was inhibited while the ascorbic acid oxidase still had high activity. Lack of oxidation of ascorbic acid by the ungerminated seed shows that the oxidase system, or at least one of its essential components, is synthesized during the course of germination. The amount of oxygen consumed in the above experiment was slightly greater than can be accounted for by the utilization of one-half mole of oxygen per mole of ascorbic acid--that is, the conversion of ascorbic acid to dehydro ascorbic acid.

NOTES ON THE BIOCHEMISTRY OF A SEED

CAROTENOIDS

There has been considerable activity in isolating and identifying carotenoids from a wide variety of natural sources. This work has been greatly aided by the application of the Tswett chromatographic adsorption technique. These studies have shown that there is spread throughout different members of the plant kingdom a large variety of carotenoids only a few of which appear to be species specific (40, 41). Moreover, within an individual plant many different carotenoids are often found, as illustrated in Strain's study on the separation of leaf xanthophylls (42). Strain finds that most green leaves contain the same xanthophylls and in approximately the same proportion.

In the animal kingdom carotenoids have been isolated from various tissues. Among the vertebrates it appears that there can be no synthesis of this type of compound and that those carotenoids found have as their origin the diet of the animal (43). However, among some of the invertebrates (especially Crustaceae) carotenoids have been isolated which are unlike any at present discovered in the plant kingdom (40).

There has been much speculation on the physiological action of these substances (44). The idea that carotenoids are in some way connected with chlorophyll in the photosynthetic process (46) is based chiefly on the following observations: (1) that the carotenoids are found in conjunction with chlorophyll in the chloroplasts of the leaf, (2) that they are strong absorbers of light energy, and (3) that yellow etiolated leaves on short exposure to light turn green. There is however no direct experimental proof that the carotenoids are in any way directly associated with the photosynthetic process. Furthermore, there is no experimental proof that the carotenoids can serve as precursors of chlorophyll or as activators for the biological synthesis of chlorophyll.

Because of the highly unsaturated nature of the compounds it has been proposed that the carotenoids act as hydrogen transfer agents for cellular oxidation processes (44). This proposition has not been experimentally confirmed. Recently papers have appeared discussing the existence in plant tissues of a carotenoid oxidase (46). It appears that the oxidation of the pigment occurs in the presence of enzyme and unsaturated fatty acid, but it is not certain at the present time whether the enzyme is necessary for the oxidation of the carotene or whether this takes place non-enzymatically by the oxidized fatty acid.

It does not appear likely that carotenoids (with the exception of vitamin A) are necessary for the efficient functioning of animal tissues. Some species have been raised carotenoid free by removal of the pigments from the diet without any apparent change in the normal functioning of the organism (43). This is in spite of the fact that some animals store relatively large amounts of certain specific carotenoids in certain tissues such as xanthophyll in the egg yolk of the chicken and ε carotene in the corpus lutem of mammals.

Recently very interesting papers have appeared describing certain carotenoid derivatives as activators in the reproduction process in algae (47).

In summary one can say that we are completely in the dark concerning any physiological role that carotenoids play in plant tissues.

The method of biological synthesis of the carotenoids is also completely unknown. The biological conversion of one carotenoid to another has not been demonstrated. Other fairly widely occurring compounds which bear some structural relationship to the carotenoids and might serve as precursors or decomposition products are phytol and the squalenes.

In line with the work on other fat soluble constituents of the tomato seed-seedling system, the following studies were undertaken in hopes of leading to a better understanding of the changes that occur among these pigments in plant tissues and their possible integration with the utilization of the stored foodstuff of the seed by the growing seedling.

CAROTENOIDS -- EXPERIMENTAL

It was early observed during this investigation on the tomato seed-seedling system that a considerable synthesis of carotenoid pigment occurred during the course of germination. Methods were set up for a closer study of the carotenoids in the seedling.

Methods of Analysis

Total Carotenoids

Extractions were made by placing a weighed portion of fresh seedlings (approximately 1 gram) in a 40 cc volume test tube homogenizer and disintegrating the tissue in the presence of 1:1 absolute ethyl alcohol--petroleum ether (b.p. 30°-60°). The tube was centrifuged and the solvent filtered into a 100 cc glass stoppered graduate cylinder. The residue was re-extracted with 1:1 absolute alcohol--petroleum ether until colorless (total volume of extracts approximately 50 cc). The filtered extracts were diluted with water until the alcohol content was 50% H_2O, thus transferring the pigments to the petroleum ether phase. The aqueous alcohol was thoroughly extracted with fresh petroleum ether. The petroleum ether was reduced to a small volume in vacuo and made up to 25 cc for total pigment estimations.

Ratio of Xanthophylls to Carotenes

The pigment from the total carotenoid estimation was partitioned between 90% ethyl alcohol and petroleum ether. The petroleum ether phase was made to 25 cc for estimation of carotenes. The alcohol phase was reduced to low volume in vacuo, water removed by additions of absolute ethanol and distillation, and finally made to 25 cc in absolute ethanol for estimation of xanthophylls.

Colorimetric Determination

The pigment was estimated colorimetrically in the Evelyn Photometer using filter 420 and 10 cc volumes in the special

calibrated test tubes. Solutions of crystalline β carotene (SMA Co.) in petroleum ether gave straight line variations of the log of the galvonometer deflection between 2 and 20 gamma per cc concentrations.

$$C = LK$$

C = Concentration of pigment in gamma per cc.
L = 2 - log of galvonometer reading.
K = 0.33 ± 0.01.

All solutions were estimated in terms of β carotene. Absorption of the various carotenoids in the region of 420 mu is sufficiently similar for the comparative purposes of this investigation.

Carotenoid Distribution in Etiolated Seedling

Carotenoid estimations were run at various times during the course of germination. The results can be seen in Table 11 and Figure 6. The ungerminated seeds contain practically no pigment. At the end of 48 hours, when the root has already broken through the seed coat, still no pigment has been manufactured. But from the end of the second day until the end of the fifth day a rapid synthesis of carotenoids takes place followed by a more gradual steady increase. Reference to the ascorbic acid section of this paper shows that the most rapid synthesis of that compound occurred somewhat earlier--during the second and third days.

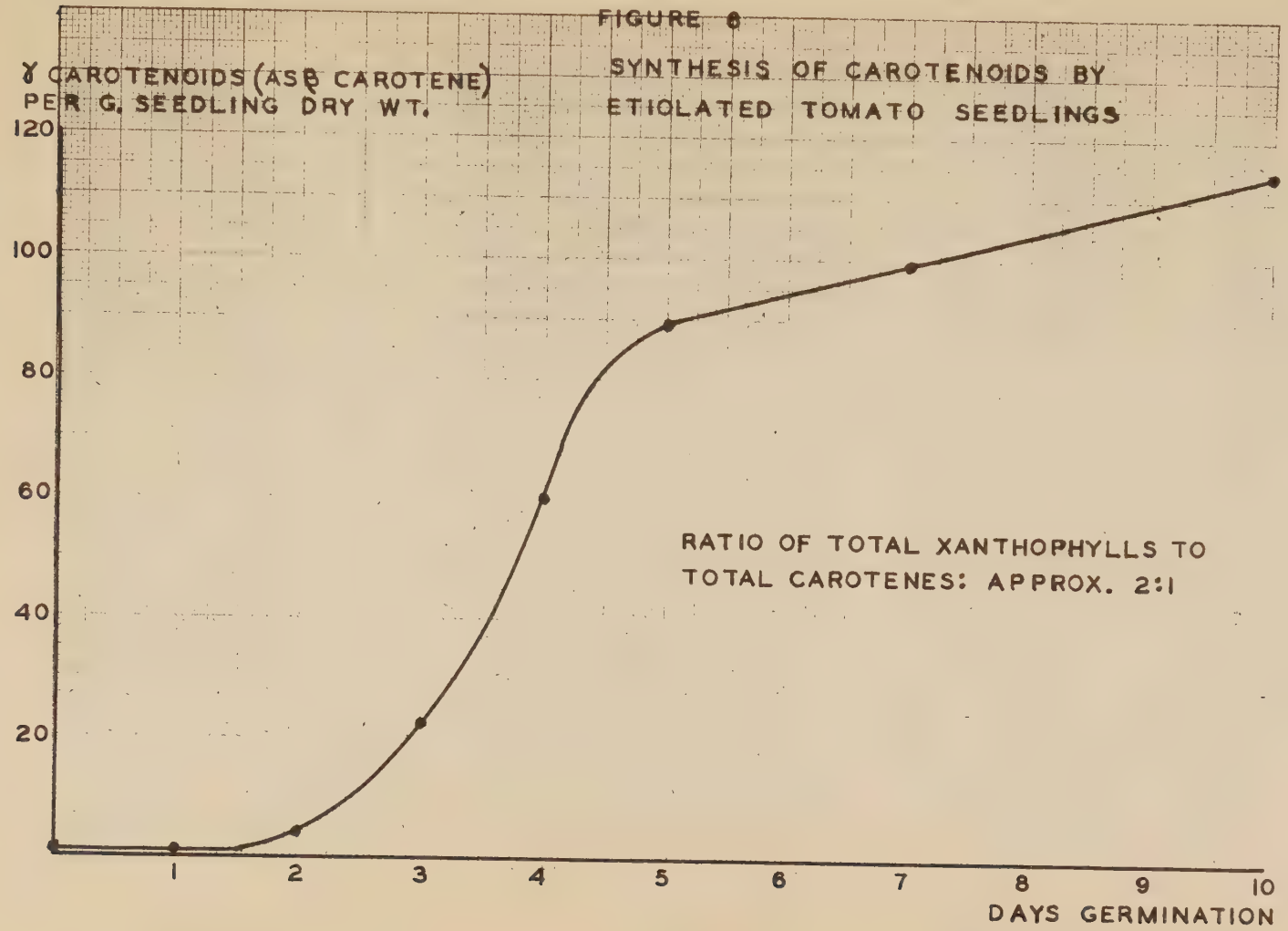
TABLE 11

CAROTENOID SYNTHESIS IN ETIOLATED TOMATO SEEDLINGS
RATIO OF XANTHOPHYLLS TO CAROTENES

Days Germination	Gamma Carotenoid per Gram Dry Seedling	Ratio Xanthophylls/Carotenes
0	2	---
1	2	---
2	4	---
3	23	2.0/1
4	60	2.0/1
5	90	2.1/1
7	100	2.1/1
10	115	2.5/1

FIGURE 6

SYNTHESIS OF CAROTENOIDS BY ETIOLATED TOMATO SEEDLINGS



Ratio of Xanthophylls to Carotenes

The oxycarotenoids (xanthophylls) can be separated from the hydrocarbon carotenes by phase distribution between 90% ethyl alcohol and petroleum ether, the xanthophylls entering the alcohol layer. This procedure was further used to analyze the pigments of the seedlings. The results are given in Table 11. At least during the early course of the germination the ratio of xanthophylls to carotenes is maintained fairly constant at approximately 2 to 1. In the phase distribution used for this analysis xanthophylls esters would remain with the carotenes in the petroleum ether. That no xanthophyll esters were present was shown by transferring the petroleum ether phase to alcohol, saponifying, removing to petroleum ether with the usual washes, and distributing again with 90% ethanol. With this procedure no appreciable loss of pigment from the petroleum ether occurred. Table 12.

TABLE 12

PRESENCE OF XANTHOPHYLL ESTERS

Sample	Carotene Fraction Gamma Carotenoid per Gram Dry Weight	
	Before Saponification	After Saponification
5 day seedling	59	56
7 day seedling	62.5	63

A further demonstration that a separation of xanthophylls from carotenes was attained by this procedure was shown by the use of an activated calcium carbonate column which adsorbs xanthophylls and no carotenes. The petroleum ether phase, after washing out traces of alcohol and drying with anhydrous sodium sulfate, passed through the column without adsorption of pigment. The alcohol phase was transferred to petroleum ether, traces of alcohol and water were removed, and on passing through an activated calcium corbonate column there was complete adsorption of pigment.

Chromatographic Separation of Carotenoids

For a further insight into the nature of the carotenoids synthesized by the seedlings, chromatographic adsorption experiments were undertaken. A column 1 x 15 cm was set up using Merck's Ppted Calcium Carbonate, heated for 6 hours at 160° just previous to using. The xanthophyll fraction from a five-day seedling preparation was adsorbed on the top of the column from a small volume of petroleum ether. The column was developed with petroleum ether. Three distinct pigment areas separated. A similar column was set up using activated alumina. The carotene fraction from five-day seedlings was strongly adsorbed from petroleum ether at the top of the column. Washing with petroleum ether did not cause a movement of pigment but with washes of petroleum ether-benzene 10:1 a separation of three bands resulted. Thus there appear to be at least six compounds present. It is entirely possible that on careful elution and rechromatographing further separation could be attained. At any rate this work shows the hetrogenious nature of both carotene and xanthophyll fractions.

Effect of Light on Synthesis of Carotenoids

A physiological integration of carotenoids and chlorophyll have been suggested because of their close physical association in the chloroplasts of green plants. Carotenoid synthesis in non-chlorophyllous seedlings (grown in dark) has just been described. It seemed worth while to compare carotenoid formation in seedlings containing chlorophyll (grown in light). The analyses for carotenoids were carried out as described at the beginning of this section except that at the time of transfer of pigment from the original seedling extract to petroleum ether the 50% alcohol was made 2% with sodium hydroxide and shaken thoroughly with the petroleum ether extracts to remove chlorophyll. Removal of chlorophyll was checked by following the 670 mu absorption band with a hand spectroscope. The results (Table 13) show that there is a significant increase in the carotenoid concentration in seedlings grown in the light. Furthermore, it is interesting to notice that the ratio of xanthophylls to carotenes is practically one as compared to a ratio of two plus in the etiolated seedlings.

TABLE 13

COMPARISON OF CAROTENOID SYNTHESIS IN LIGHT AND DARK

Days Germination	Gamma Carotenoid per Gram Dry Seedling		Ratio Xanthophylls/Carotenes	
	Light	Dark	Light	Dark
5	157	90	1.1/1	2.1/1
10	195	115	1.1/1	2.5/1

Enzymatic Oxidation of Carotenoids

It was observed that when the fresh seedlings were crushed the carotenoid pigments rapidly bleached. In order to determine whether this decolorization of the fatty pigments was directly or indirectly catalyzed by enzyme action the following experiment was set up. A portion of the fresh five-day seedlings was placed in the bottom of the homogenizer tube, thoroughly macerated with the bottom of the homogenizing cylinder and allowed to stand for 15 minutes at room temperature before extraction for pigment with the alcohol - petroleum ether. Another portion of seedlings was placed in the tube and crushed while the tube was immersed in boiling water for five minutes. It was then cooled, let stand in the air for 15 minutes, and extracted for pigment analysis. A third portion of the same batch of five-day seedlings was extracted directly as in the regular pigment analysis.

All the pigment remained in the heated preparation, whereas practically all the pigment disappeared in the non-heated sample. Table 14. Thus it appears likely that protein heat denaturation removed from the crushed seedlings a catalyst for the bleaching of carotenoids. Obviously this catalytic system is not specific for any single carotenoid.

TABLE 14

BLEACHING OF CAROTENOIDS BY TOMATO SEEDLING TISSUE

Sample	Gamma Carotenoid per Gram Dry Seedling
5 day seedlings, regular extraction	60
Crushed, 3' at 100°, cooled, 15' in air before extraction	62
Crushed, 15' in air before extraction	6.5

STEROLS

For a long time it has been known that plants contain a sterol called sitosterol, closely related to the most commonly occurring animal sterol, cholesterol. In 1906 it was shown that soybeans contain stigmasterol (48), a more unsaturated form than the sitosterol then known. In 1927 Anderson and co-workers (49), working with various seed oils, showed that the original sitosterol was in reality a mixture of several sterols. They separated α, β, γ, and dihydro sitosterol fractions. Fernholz (1936, 1939) (50, 51) separated two more sterols from the α or more soluble fraction.

The sitosterols although fairly widely occurring are not the exclusive sterol components of the plant kingdom. A number of others have been demonstrated, for example: fucosterol from algae (52), stigmasterol from soybean (53), spinasterol from spinach and other sources (54), brassicasterol from rape seed oil (55), and special sugar cane sterols (56). It is evident now that there is no certain group of sterols existent in all plant tissues. However, little can be said at this time on the general distribution of these compounds in the plant kingdom. There is no evidence available on the types and amounts of sterol in the various tissues of any given plant.

Due chiefly to the work of Fernholz, Wallis, and co-workers the structure of a number of the phytosterols has been established. β and γ sitosterols, stigmasterol and brassicasterol all have the same ring nucleus as cholesterol but differ in the side chains. Stigmasterol and brassicasterol have an unsaturated bond in the side chain, stigmasterol being the 22 dehydro β sitosterol. The α sitosterols have two unsaturated bonds in the ring system. A good review on the chemistry of these compounds is given by Heilbron, 1940 (57).

As yet no light has been thrown on the metabolism or physiological functions of the sterols in plants.

The few sterol isolations that have been made have not been such as to give information on the natural state of occurrence. The routine saponification techniques break up naturally existing ester linkages. The author has shown that the majority of the sterols of wheat germ oil exist naturally in a combined form (58). Kraybill and co-workers have demonstrated sterol glucosides in soybean and cottonseed oils (59).

Practically nothing is known concerning methods of synthesis or changes of the sterol molecule in living plant tissues. McLachlan (60) has reported on sterols during germination of soybeans. He finds evidence of some synthesis and some esterification during this period of the plant growth. He gives no data on the nature of the sterol or the nature of the ester formed.

There is no evidence for the general existence in plants of sterols of the type showing hormonal activity in animals, although that the basic structure is the same has been adequately confirmed by the conversion of sitosterol and stigmasterol to sex hormones (61). It is interesting, however, that plant tissues do contain sterol which can be made Vitamin D active by irradiation (62). These have not been isolated. Pure sitosterol or stigmasterol show no provitamin D activity. However, on forming the 7 dehydro derivatives of these compounds they can be made antirachitic on irradiation (63). From the present knowledge of the structure of provitamin D sterols, it seems fairly certain that there must exist in plant tissues sterols of the 3 OH, 4-5 , 7-8 type.

It appears that seed oils in general contain sterol as a major constituent of their non-glyceride fractions. Does this sterol play a role in the utilization of the stored food of the seed by the growing seedling? By analogy with the potent biological effects of sterols in animals it would seem worth while to follow qualitatively and quantitatively the occurrence and changes of these compounds in the seed-seedling system under observation. This is the concern of the experimental work to follow.

STEROL -- EXPERIMENTAL

Extraction of Tomato Seed Oil

One kilo of clean tomato seeds were ground to 50 mesh in a Wiley Mill and extracted in a large soxelet for six hours with benzene. The solvent was distilled off in vacuo. Yield: 230 grams of bland tasting clear light brown oil. (If oil is prepared from the seed in the cold, it is practically colorless. Heating causes a darkening. This color remains in the saponifiable portion and is therefore probably due to changes in the glycerides.)

The oil was quite low in free fatty acid. Analysis: By titrating the oil in alcohol-benzene with 0.1 N NaOH to phenolphthalein and expressed as per cent oleic acid.

Wt. oil	4.500 grams
0.1 N NaOH	1.50 cc

% Free Fatty Acid 0.9

Non-Saponifiable Fraction of Oil

Quantitative non-saponifiable estimations were carried out by the method of the American Oil Chemists Society (65). Particular care in the details must be followed in this analysis for accurate and consistent results.

Sample Analysis:

Wt. Oil	4.6943 grams
Yield Non-sap.	0.0474 grams

% Non-Saponifiable 1.0

The non-saponifiable fraction is white, waxy in appearance. From various attempts to isolate sterol it appeared that only a small per cent of the non-saponifiable fraction consisted of sterol.

Extraction of Sterol

Because of the difficulty in thoroughly extracting large volumes of soap solutions and also in order to determine the possible presence of sterol esters, the seed oil instead of being saponified was extracted directly with 95% ethyl alcohol. Only a small per cent of the triglyceride is soluble in alcohol and previous experience by the author with wheat germ oil had shown that the wheat sterols could be crystallized directly from a concentrated alcohol extract of the oil. 200 grams of the benzene extracted oil were extracted repeatedly with a total of two liters of 95% ethanol. Extraction was carried out by vigorous shaking in stoppered 250 cc centrifuge bottles with subsequent centrifugation for complete separation of the alcohol and oil layers. 9.0 grams or 4.5% of the original oil was soluble in the alcohol. The alcohol was concentrated to a small volume and left in the cold for crystallization of sterol.

However, persistent cloudiness in concentrated alcoholic solutions prevented a separation of crystalline sterol. Therefore, the alcohol soluble oil was saponified with 10% methanolic KOH and the non-saponifiable picked up in petroleum ether from the soap solution. The petroleum ether was removed in vacuo. Yield non-saponifiable 1.5 gram. The residue was taken up in a minimum of hot 95% ethanol and set aside for crystallization of sterol.

Tomato Seed Sterol

A total of 100 mg of crystalline sterol was isolated after working up of filtrates. After recrystallization from alcohol the sterol melted sharply at 137°.

The crystals gave positive Salkowski and Lieberman-Burchard color tests.

Optical Rotation of Free Sterol

23.3 mg per 2.0 cc chloroform, l = 1dcm

$[\alpha]_D = -34$

Acetate

To 45 mg of sterol was added 3 cc of acetic anhydride and the mixture refluxed for 30 minutes. On cooling crystals of the acetate formed. These were separated and recrystallized from ethyl alcohol. M.P. 126°.

3, 5 Dinitrobenzoate

The acetate was saponified and the free sterol picked up from aqueous alcohol into petroleum ether. The petroleum ether was evaporated from a small test tube and 200 mg of freshly prepared m dinitrobenzoyl chloride and 1.5 cc of pyridine were added and the mixture refluxed for 15 minutes. The mixture was diluted with water and the crude ester filtered and washed with water. The ester was crystallized from benzene-ethanol. M.P. of pale yellow needles 207°.

The constants given agree very well for those reported for β sitosterol and are sufficiently different from those of the other phytosterols that there is no doubt about the identity of the compound isolated. Table 15. See also table in (66) page 163.

TABLE 15

COMPARISON OF β SITOSTEROL AND TOMATO SEED STEROL

Sample	Sterol mp	Sterol $[\alpha]_D$	Acetate mp	3.5 Dinitro-Benzoate mp
β Sitosterol (66)	136-7	-36.6	125-6	202-3
Constants (67)	135-5.5	-34.2	126-7	207-9
(68)	136-7	-31.5	122-3	---
Tomato Seed Sterol	137	-34	126	207

Working up of the filtrates from the sterol isolation failed to show the presence of the α more soluble sterols which have been isolated from some seed germ oils such as rye and wheat. Also from the apparent homogeneity of the preparation it appears unlikely that there is any significant amount of stigmasterol

present. Thus it appears that at least the greater part of the sterol of the tomato seed oil consists of β sitosterol. This is similar to the results found by Wallis and Chakrovorty with cottonseed oil (66).

Seedling Sterol

A small amount of sterol melting at 137° and evidently the same sitosterol was isolated from 7-day seedling oil. However, the problems as to whether there is a gain or loss of sterol during the course of germination or whether there are any changes such as esterifications has not as yet been thoroughly investigated.

Provitamin D Sterols in Tomato Seed

Rat bioassays were carried out to determine the presence of provitamin D compounds in the tomato seed. Three-week-old male rats were kept in carefully cleaned cages in a room without sunlight and fed the Steenbock rickets producing diet #3145 for two weeks. The rats were then fed the following diets for ten days. On the eleventh day they were sacrificed and inspected for degree of rickets by the line test (69). The rats were divided into five groups.

1. 6 rats. Controls, Steenbock diet alone.
2. 6 rats. Rachitic diet ad libitum. 0.1 cc per day each rat - one part irradiated tomato seed oil dissolved in three parts inert sesame oil.
3. 6 rats. Rachitic diet ad libitum plus 0.1 cc per day per rat of irradiated tomato seed oil.
4. 6 rats. Rachitic diet plus 0.4 grams of irradiated tomato seed meal per rat per day.
5. 5 rats. Rachitic diet plus 0.4 grams of non-irradiated tomato seed meal per rat per day.

Rats were given weighed portions of tomato seed meal in separate dishes. There was no difficulty about their eating the meal completely.

Tomato seed oil was administered into the esophagus of the rat from a blunt-pointed syringe.

Irradiations were carried out by placing the material 40 cm from a Cooper-Hewitt mercury vapor ultra violet lamp and given an exposure of 35 minutes.

The results of these bioassays are given in Table 16.

TABLE 16

BIOASSAY FOR VITAMIN D ACTIVE CONSTITUENTS
OF TOMATO SEED

Samples Assayed	Rat No.	Rat Weights					Line Test
		12/8	12/15	12/22	12/26	12/31	
(1) Controls	22	35	41	52	53	58	R
	15	32	38	47	46	46	R
	13	32	35	45	47	53	R
	19	32	37	45	48	47	R
	9	28	30	34	31	31	R
(2) 0.015 cc of irrad. tomato oil per day	10	34	36	40	41	43	1+
	29	31	35	40	43	45	1+
	17	36	44	52	53	56	1 - 2+
	5	27	32	42	43	49	1 - 2+
	12	34	42	49	50	54	1+
	21	36	44	50	53	54	2+
(3) 0.1 cc/day of irrad. tomato seed oil	2	35	42	42	44	46	3+
	6	32	39	42	42	44	3+
	27	40	49	55	56	58	3+
	23	33	41	48	48	54	3+
	18	40	50	60	60	66	3+
	26	29	34	40	42	47	3+
(4) 0.4 grams of irrad. tomato seed meal/day	16	36	42	49	49	54	3+
	25	34	37	42	44	48	3+
	11	35	42	52	55	62	3+
	20	33	38	45	48	50	4+
	28	33	42	48	50	54	3 - 4+
	30	26	30	33	35	38	4+
(5) 0.4 grams of non-irradiated tomato seed meal/day	3	35	44	54	57	63	R
	8	30	36	43	40	46	R
	14	34	40	48	56	54	R
	7	35	40	49	50	56	R
	24	26	32	38	38	40	R

Steenbock diet #3145 and water ad libitum.

Assay samples fed during last ten days.

1+ ——→ 5+, slight to complete cure by bone line test.

R = complete rickets.

Discussion of Results

Complete florid rickets developed in all controls and in all rats receiving non-irradiated tomato seed meal. Animals did not lose weight during assay period. Checks were excellent among individual members of a group. Activity per unit seed oil compared well with a previous experiment. Therefore, the results can be considered valid.

The tomato seed contains pro-vitamin D in its oil fraction (Group 2).

Non-irradiated meal has no antirachitic properties (Group 5).

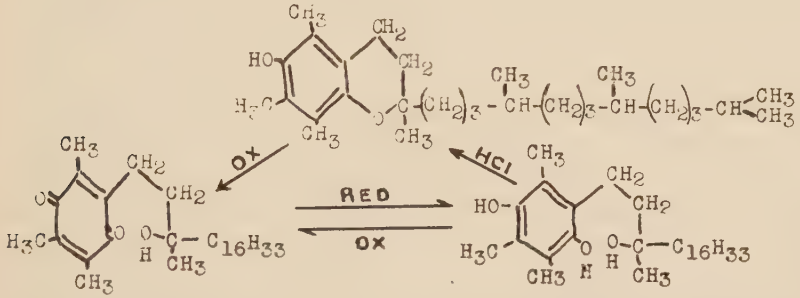
Irradiated meal showed vitamin D activity equivalent to its oil content (25%). Compare Groups 3 and 4.

The tomato seed contained no other substances (cf. inorganic salts) which effected the development of rickets (Group 5).

A one plus cure is equivalent by this method to one Steenbock Rat Unit or approximately 3.3 International Units (70) (fed over 10-day period). Therefore, the irradiated tomato seed oil contains approximately 15 Int. Units per gram (sp. gr. oil 0.9). One Int. Unit is equal to 0.025 gamma calciferol. Therefore, if we assume a 100% activation of the provitamin by irradiation, then, one gram of oil contains 0.37 gamma provitamin D sterol expressed as calciferol. Since the seed oil has approximately 0.1% total sterol, the provitamin D sterol, then, represents only about 3 to 4 hundredths of a per cent of the total sterol fraction.

TOCOPHEROLS

Tocopherol can be oxidized to a tocoquinone and reduced back again according to the scheme (71):



It has been suggested that this sort of oxidation-reduction might take place in vivo and thus the tocopherol could act as a respiration catalyst. However, this line of reasoning was made unlikely when the tocoquinone was found to be biologically inactive (72). Furthermore, no respiration effects have been shown in tocopherol deficient tissues.

Mattill and co-workers have carried out a number of experiments demonstrating the antioxidant activity of the tocopherols. Mattill has suggested that a physiological function of the compounds may be the prevention of the rancidity type of oxidative changes in fatty acids (73).

It has been assumed that tocopherols are widely distributed in the plant kingdom. This assumption rests chiefly on experience gained in trying to prepare tocopherol free diets for animal experimentation. There have been few careful quantitative studies on the distribution of this compound (74). This is largely due to the fact that the bioassay is very difficult, time consuming, expensive, and subject under the best of conditions to a high percentage of error. There is another difficulty from the viewpoint of the plant physiologist in interpreting those analyses which have been reported. Too often the investigator uses an oil which

has been purchased on the market or taken from the laboratory shelf--a preparation from the uncertain past. The reports in these cases are practically valueless in interpreting the natural distribution of the compound in question. The reasons for this are: (1) The methods of extraction--heat in pressing, solvent used, etc.--cause great variation in removal of the tocopherol content of plant tissues; (2) oil refining techniques cause large losses (alkali treatment) and removal (adsorbents) of the tocopherol; (3) the tocopherol in an oil can be more or less rapidly destroyed after removal of the oil from the plant tissue depending on a number of factors, development of rancidity, chemistry of the oil (unsaturation, antioxidants, pro-oxidants), and degree of exposure to light and air. About all that can be said concerning the distribution is that of those substances investigated the germ of wheat seems to have the highest concentration. It is present in some succulents (of lettuce), and it is fairly generally distributed in seed oils.

The phenolic hydroxyl lends this compound its strong reducing properties. Because of this group one must also suspect that the compound could exist naturally in the esterfied condition. Karrer (75) believes that because of the non-carotenoid reducing action of whole oils--which he considers due to tocopherol--the compound must exist in an uncombined state.

At least in some species of animals tocopherol plays the role of a very essential and potent biocatalyst. For example, a rat without tocopherol will not only show a specific destruction of the gonads but will lose weight, develop paralysis, undergo severe muscular degeneration, etc. The line of reasoning of this investigation has been that if a substance has been shown to be a potent biocatalyst for animal tissues it is worth while investigating for a possible role in plant tissues. Therefore, in the experimental work to follow, the presence and state of tocopherol in the tomato seed are investigated and any changes in the metabolism of this compound during the germination period are looked for.

TOCOPHEROL -- EXPERIMENTAL

Methods

Tocopherol estimations were carried out by the procedure devised by the author (Master's thesis, University of Chicago, 1940). The technique involves a combination of two tests. First, measurement of the reduction of ferric ion by tocopherol under certain specific conditions, the ferrous formed being estimated colorimetrically with $\alpha\,\alpha'$ dipyridyl. Second, colorimetric measurement of the red orthoquinone formed from tocopherol by oxidation with nitric acid. Analyses are performed on whole oil extracts of the seeds or fresh seedlings. Saponification is avoided because of the destruction of tocopherol caused by alkali. Tests on fresh seedlings were made on aliquots of the petroleum ether-alcohol extract as described in the carotenoid section of this paper (p. 30). The chief interfering material in this determination is carotenoid pigment. It gives a slight extraneous color in the nitric acid test but interferes markedly in the ferric chloride test. Pure crystalline carotenoids (xanthophyll and b carotene - SMA Co.) reduce ferric ion in the presence of dipyridyl. For example, the readings given in the Evelyn Phototometer by 4 gamma per cc concentrations in absolute alcohol under the conditions of the ferric chloride test were:

TABLE 17

COMPARISON OF THE REDUCTION OF FERRIC BY TOCOPHEROL AND CAROTENOIDS

Sample	Galvonometer Deflection from Blank on Reagents
Tocopherol	45
Xanthophyll	52
Carotene	53

Readings 5 minutes after mixing reagents
Filter 520

There are no carotenoids in the ungerminated tomato seed (see p. 35). However, in the seedling enough carotenoid pigment is synthesized to interfere with the tocopherol determination. It has been proposed to separate tocopherol from carotenoids by adsorption. However, no adsorbent investigated has proved practical for a quantitative separation. The interference in the seedling tissues can be very easily avoided by taking advantage of the carotene oxidase system as described on page 37. The seedlings are thoroughly crushed and allowed to stand in the air for 10 minutes before extraction. During this time the carotenoids are oxidized to colorless forms which do not interfere in the tocopherol test. Recoveries of added synthetic tocopherol show that the tocopherol itself is not oxidized by this procedure.

Results

It is demonstrated that the tomato seed contains significant amounts of tocopherol--0.7 to 0.8 mg per gram seed dry weight, or approximately 0.3% of the seed oil or approximately 30% of the non-saponifiable fraction of the oil. The concentration of this compound in terms of dry weight seedling remains fairly constant during the germination period.

TABLE 18

TOCOPHEROL DISTRIBUTION IN TOMATO SEED-SEEDLING

Sample	Tocopherol - mg/g Dry Weight	
	Nitric Acid Test	Ferric Cloride Test
Ungerminated seed	0.72	0.75
3 day seedling	0.70	0.73
5 day, before oxidation of carotene	....	1.02
5 day, after oxidation of carotene	....	0.75
5 day, after oxidation +0.5 mg/g of tocopherol	....	1.25
10 day seedling	0.65	0.68

A further qualitative demonstration of the presence of tocopherol in the tomato seed was made by demonstrating the 294 mu tocopherol absorption maximum in an extract of the seed oil. Various authors have pointed out the difficulty of demonstrating this absorption maximum, except in very highly purified extracts, because of the strong general absorption in this region by other constituents of vegetable oils (77). However, a satisfactory extract of the seed oil was made in the following simple manner. The oil was shaken with absolute ethyl alcohol at room temperature and then allowed to stand for a couple of days in the cold room at -8° for separation from the alcohol of glycerides, sterols, and other possible interfering materials. The resulting alcohol solution was then photographed in a B. and L. Quartz Spectrograph (hydrogen arc as light source) and showed a distinct absorption maximum at 294 millimicrons.

SUMMARY AND DISCUSSION

It is fascinating to observe a seed, an apparently inert mass, merely by the addition of water become an actively matabolizing growing organism. The curiosity of the biochemist is whetted to learn something of the chemical changes involved in this process. Therefore, the author initiated this work, choosing the tomato seed as the particular subject of investigation. The presence and activities of various chemicals were examined which by analogy with their action in better studied tissues might play important roles in the seed-seedling conversion. As a start towards the unraveling of the seed-seedling riddles, the following results have been obtained.

Convenient methods of handling of the seeds and seedlings for analytical work have been established.

A transformation of major interest involves the disappearance of oil in the seedling. Analyses showed a drop in oil content from 26% in the seed to 4% in the 15-day seedling with an unexplained temporary increase shortly after the start of germination.

After the beginning of growth intact seedlings and finely disintegrated seedling suspensions consume oxygen at the rate of 2 to 3 cmm oxygen per mg tissue per hour. Temperature and pH activation curves are given for this O_2 uptake. CO_2 is evolved by the seedling at such a rate as to give a very low R.Q. value of approximately 0.3. Such a low R.Q. might be explained by a conversion of fat to carbohydrate within the seedling.

A major part of the oxygen consumption is not inhibited by as high a concentration as 0.01 M cyanide, thus excluding the cytochrome system from the activation of this part of the oxygen. Spectroscopic search for the 550 mu absorption band of reduced cytochrome c was negative. Thunberg tests on the decolorization of methylene blue with seedling suspensions containing added suc-

cinate gave negative results for succinic-dehydrogenase. Thus it appears that such important hydrogen transfer agents for animal tissues as the cytochromes and succinic-dehydrogenase are either absent or present in very small quantities in the tomato seedling. This is not generally true for plant tissues. Both succinic-dehydrogenase and cytochrome were found in wheat. It is suggested that this difference may be related to the fact that one tissue is catabolizing large amounts of fat while the other system is handling chiefly carbohydrate.

An unusually high concentration of thiamin--17 gamma per gram--was found in the tomato seed. This high value was confirmed by assay on polyneuritic rats. The total thiamin concentration fell somewhat during the germination period. No cocarboxylase was present in the ungerminated seed. However, the diphosphothiamin appeared in the seedling to the extent of approximately 1 gamma per gram, showing that some of the thiamin had been phosphorylated early in the germination. Why is it that there is such a large ratio of free thiamin to DPT (about 15:1) in the growing seedling when only the DPT is supposed to be functionally active?

The DPT acts with a carboxylase in the seedlings to split off CO_2 from pyruvic acid. The ungerminated seeds do not attack pyruvic acid, but that the carboxylase enzyme is present in the ungerminated seed is evident from the fact that decarboxylation of pyruvate resulted from the addition of DPT to the seeds.

The pyruvate utilization of the seedlings is of a small order of magnitude, approximately 0.03 millimoles of pyruvate being consumed per gram of tissue per hour. All of the pyruvate utilized appeared to be decarboxylated (no oxidation). About a half of the pyruvate disappearing was recovered from the tissue suspensions as acetaldehyde. Wheat and corn seedlings were found to have much more active pyruvate decarboxylating systems than the tomato seedlings. Here again is a possible differentiation between a fat and a starch type of seedling. All of the pyruvate utilized in a corn germ suspension was recovered as acetaldehyde.

The dry seeds contain no ascorbic acid. However, shortly after the start of growth there is a very rapid synthesis of this compound reaching a maximum of 0.54 mg per gram between the third and fourth days of germination. In fresh seedlings no evidence was found for the existence of either dehydroascorbic acid or

bound ascorbic. Crushing the seedling tissues alters the living systems so that the ascorbic acid present is rapidly oxidized. This oxidation was found to be enzymic in character, and furthermore it was shown that the oxidase was not present in the ungerminated seed but synthesized at the start of the germination period.

No significant difference in ascorbic acid content was observed between etiolated and chlorophyllous seedlings. An unavoidable observation was that fatty extracts developed considerable yellow color during the course of germination. The ungerminated seed contains negligible amounts of carotenoid pigment--however a large synthesis takes place early in the germination period reaching values of about 100 gamma per gram dry weight of seedling for total carotenoid concentration. This carotenoid production was followed quantitatively and qualitatively in terms of the ratio of xanthophylls to carotenes (found to be approximately 2:1). Both xanthophyll and carotene fractions were shown by absorption experiments to contain a multiplicity of components.

Comparisons between etiolated and chlorophyllous seedlings indicated that there was an increased synthesis of carotenoid in the light and furthermore that this was associated with a lower ratio of xanthophylls to carotenes.

It was noticed that on crushing the fresh seedling tissues the yellow pigment rapidly bleached colorless. Heat inhibition experiments indicated that this process was enzyme catalyzed.

A comparatively large amount of tocopherol was found in the seed--0.7 mg per gram. Its concentration remained fairly constant during the germination period. A method was devised for the analysis of this compound in the presence of interfering carotenoids by making use of the carotenoid oxidase system found in the seedlings. The presence of tocopherol was confirmed qualitatively by demonstrating the absorption maximum at 294 millimicrons.

Sterol was separated from the tomato seed oil. Practically all of the sterol fraction consists of the single compound b sitosterol. Optical rotations, and melting points on free sterol, acetate, and dinitrobenzoate, conclusively identified this sterol. The results are similar to those found by other workers with another fatty seed (cottonseed) and unlike results

with the starchy grains (rye, corn, wheat) which have been shown to contain a hetrogenious mixture of sterols. Bioassay on rachitic rats showed the presence of a provitamin D sterol, present in a concentration of about 0.03% of the total sterol.

Tomato seed oil contains one per cent of non-saponifiable material. Of this non-saponifiable fraction, approximately 30% is tocopherol and about 10% sterol.

* * * * * * *

It is felt that one of the more important contributions of a research such as this is the establishment of methods and the gaining of experience in handling a certain type of tissue so that further research on the subject will be increasingly more profitable. The work already started suggests many likely fields of continuation. A few possibilities will be mentioned here.

A closer scrutiny of the fate of the stored oil of the seed has already been undertaken and has resulted in the preparation of dry defatted seedling powders which will actively hydrolyze fatty triglycerides and oxidize fatty extracts of the seedling. This work is being extended.

A further examination of the non-saponifiable components of the oil is in order. It is only of secondary importance to have found out that the seed oil contains about 0.1% b sitosterol and 0.3% tocopherol. However, it is important that these constituents have been quantitatively identified so that their possible integration with the oil metabolism of the seedling can be checked and samples used for study in isolated metabolizing systems.

An obvious follow-up of the pyruvate metabolism experiments is to determine the fate of the acetaldehyde.

The finding of DPT synthesis from thiamin raises the question of the method of phosphorylation.

The presence of such an active carboxylase as found in the wheat and corn germs suggests isolations and comparisons with the carboxylase which has been prepared from yeast.

Aside from establishing facts for correlation with changes taking place in the seed-seedling system, the work on ascorbic

acid and carotenoids lays a foundation and suggests methods for studying the details of the biological synthesis of these compounds.

The general respiration studies can be expanded along many lines following standard procedures developed with animal tissues.

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